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Dual-specificity phosphatase 23 mediates GCM1 dephosphorylation and activation

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ABSTRACT

Glial cells missing homolog 1 (GCM1) is a transcription factor essential for placental development. GCM1 promotes syncytiotrophoblast formation and placental vasculogenesis by activating fusogenic and proangiogenic gene expression in placenta. GCM1 activity is regulated by multiple post-translational modifications. The cAMP/PKA-signaling pathway promotes CBP-mediated GCM1 acetylation and stabilizes GCM1, whereas hypoxia-induced GSK-3β-mediated phosphorylation of Ser322 causes GCM1 ubiquitination and degradation. How and whether complex modifications of GCM1 are coordinated is not known. Here we show that the interaction of GCM1 and dual-specificity phosphatase 23 (DUSP23) is enhanced by PKA-dependent phosphorylation of GCM1 on Ser269 and Ser275. The recruitment of DUSP23 reverses GSK-3β-mediated Ser322 phosphorylation, which in turn promotes GCM1 acetylation, stabilization and activation. Supporting a central role in coordinating GCM1 modifications, knockdown of DUSP23 suppressed GCM1 target gene expression and placental cell fusion. Our study identifies DUSP23 as a novel factor that promotes placental cell fusion and reveals a complex regulation of GCM1 activity by coordinated phosphorylation, dephosphorylation and acetylation.

INTRODUCTION

Glial cells missing homolog 1 (GCM1, also known as GCMa) is a key transcription factor in placental development. Genetic ablation of mouse GCM1 results in embryonic lethality due to failure of labyrinth layer formation and fusion of trophoblasts to syncytiotrophoblasts (1,2). Correlatively, mouse GCM1 has been shown to regulate expression of integrin-α4 and Rb1 genes, which play important roles in the development of syncytiotrophoblast and labyrinth (3). Although GCM1 is primarily expressed in placenta, its expression has also been reported in mouse kidney and thymus (4). The physiological functions of GCM1 in kidney and thymus are not known. Interestingly, *in utero* injection of GCM1-expressing retrovirus into mouse embryonic brains indicates that GCM1 promotes the generation of a minor population of glial cells (5).

Human GCM1 positively regulates syncytin-1 and placental growth factor (PGF) gene expression, which is critical for trophoblastic fusion and placental vasculogenesis (6–9). Clinically, expression of GCM1 as well as its target genes, syncytin-1 and PGF, is decreased in preeclampsia, which is a prevalent pregnancy disorder, and in hypoxic placental cells (10–12). Since hypoxia caused by incomplete trophoblast invasion and impaired spiral arterial remodeling is associated with preeclampsia (13,14), we have recently investigated the molecular mechanism by which hypoxia decreases GCM1 expression. We demonstrated that GSK-3β mediates phosphorylation of GCM1 on Ser322, which is recognized by the F-box protein, FBW2, to promote GCM1 ubiquitination and degradation (15,16). In addition, GSK-3β is activated to further decrease GCM1 stability in placental cells subject to hypoxia. Therefore, enhanced phosphorylation of Ser322 by GSK-3β suppresses GCM1 activity in placenta, which may contribute to the development of preeclampsia.

Our previous studies have demonstrated that GCM1 activity can be regulated by ubiquitination, acetylation and sumoylation (16–18). Indeed, FBW2, CBP and Ubc9 interact with GCM1 to promote ubiquitination,

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acetylation and sumoylation of GCM1, respectively. Although these modifications all affect GCM1 stability and transcriptional activity, whether they are mechanistically connected is not known. Here we identify dual-specificity phosphatase 23 (DUSP23), which belongs to the type-I cysteine-based protein tyrosine phosphatase (PTP) superfamily (19), as a new GCM1-associated protein that mediates Ser322 dephosphorylation and thereby prolongs the half-life of GCM1. We further demonstrate that DUSP23-mediated GCM1 dephosphorylation is a prerequisite step for further GCM1 acetylation by CBP, which positively regulates GCM1. In addition, knockdown of DUSP23 suppresses GCM1 target gene expression and placental cell fusion, supporting a critical role of DUSP23 in regulation of GCM1 modification and function. Collectively, our study delineates the mechanism underlying GCM1 dephosphorylation and suggests a cascade of coordinated phosphorylation, dephosphorylation and acetylation events that is critical for controlling GCM1 activity in the regulation of placental cell fusion.

MATERIALS AND METHODS

Plasmid constructs

The pGal4-FLAG empty expression plasmid and the pGal4-GCM1-FLAG expression plasmids harboring full-length or truncated GCM1 have been described previously (17). Human GCM1 cDNA fragment with an N-terminal triple HA tag or a C-terminal triple FLAG tag was subcloned into a pEF1 expression vector under control of EF1 promoter to generate the pHA-GCM1 or pGCM1-FLAG expression plasmid. The pHA-GCM1SSAA expression plasmid was similar to pHA-GCM1 except that the GCM1 cDNA harbored Ser269-to-Ala and Ser275-to-Ala mutations. The pHA-GCM1SSEE expression plasmid harbored Ser269-to-Glu and Ser275-to-Glu mutations in the GCM1 cDNA. The pDUSP23-Myc or pDUSP23-FLAG expression plasmid was constructed by subcloning human DUSP23 cDNA fragment with a C-terminal quadruple Myc tag or triple FLAG tag into the pEF1 expression vector. The pDUSP23DACS-Myc expression plasmid was similar to pDUSP23-Myc except that the DUSP23 cDNA harbored Asp65-to-Ala and Cys95-to-Ser mutations in the active site of DUSP23. The reporter plasmid, $p(GBS)_4E1BLuc$, which contains four copies of GCM1-binding site, has been described previously (17).

Cell culture, transfection and lentivirus transduction

293T and BeWo cells were obtained from the American Type Culture Collection (Manassas, VA). BeWo31 cells which stably expressing HA-tagged GCM1 were established as previously described (16). 293T cells were maintained at 37°C in minimal essential medium alpha medium, with 10% fetal bovine serum (FBS), 100 mg/ml streptomycin and 100 U/ml penicillin. BeWo and BeWo31 were maintained at 37°C in F-12K medium supplemented with 15% FBS and the aforementioned antibiotics. Because the expression of HA-tagged GCM1 in BeWo31 cells is under control of CMV promoter whose activity is not affected by hypoxia (15), BeWo31 cells provided a useful system to investigate hypoxia-induced GCM1 phosphorylation and degradation in this study. Villous cytotrophoblast (CTB) cells from term placentas were prepared and cultured as previously described (15). For transient expression, 293T cells were transfected with expression plasmid(s) using the calcium phosphate coprecipitation method or the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Luciferase assays were performed as previously described (20). Specific luciferase activities were normalized by protein concentration. Protein concentrations were measured by using the BCA protein assay kit (Pierce, Rockford, IL). Hypoxia was achieved by exposing cells to 1% O_2, 5% CO_2 and 94% N_2 in a multigas incubator (Astec, Fukuoka, Japan), whereas normoxia was achieved with 20% O_2, 5% CO_2 and balanced N_2.

Recombinant lentivirus strain expressing DUSP23-Myc or DUSP23DACS-Myc was prepared using a modified pCDH expression vector (SBI, Mountain View, CA) harboring a puromycin selection cassette. BeWo31 or 293T cells were infected with the lentivirus strains harboring empty vector, DUSP23-Myc and DUSP23DACS-Myc, respectively, followed by antibiotic selection using 100 μg/ml puromycin. Puromycin-resistant clones were pooled for GCM1 stability, phosphorylation and acetylation studies in this study. For RNA interference, a pLKO.1-Puro lentiviral construct harboring a scramble (Addgene plasmid 1864) or DUSP23 shRNA (5′-GCTGA AATCCGACGACTACGA-3′, National RNAi Core Facility of Taiwan) was packed into recombinant lentivirus strain for establishing stable 293T, BeWo and BeWo31 cells expressing scramble or DUSP23 shRNA.

Immunoprecipitation and immunoblotting

For GCM1 and DUSP23 interaction study, 293T cells were transfected with pHA-GCM1 and pDUSP23-FLAG for 48 h. Cells were then harvested in the lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 10% glycerol, 0.5% NP-40, 5 mM NaF, 1 mM Na_3VO_4 and a protease inhibitor cocktail (Sigma, St. Louis, MO) for immunoprecipitation and immunoblotting with anti-HA or anti-FLAG mAb (Sigma). Interaction between GCM1 and DUSP23 in BeWo cells was analyzed in similar conditions using GCM1 and DUSP23 antibodies. Human DUSP23 antibody was raised in guinea pigs using a His-tagged DUSP23 antigen prepared in the pET21-BL21(DE3) expression system (Novagen, Madison, WI). To study the effect of DUSP23 on GCM1 ubiquitination, 293T cells were transfected with pGCM1-FLAG, pHA-Ub and pUDSP23-Myc or pDUSP23DACS-Myc, followed by treatment with MG132. Analysis of the ubiquitinated GCM1 was performed by immunoprecipitation with FLAG mAb and immunoblotting with HA mAb as previously described (16).

Mapping the DUSP23-interacting domain in GCM1

To map the GCM1 domain that interacts with DUSP23, pull-down assays were performed. In brief, 293T cells were

transfected with pGal4-FLAG, full-length, or the indicated deletion mutant pGal4-GCM1-FLAG plasmid. At 48 h post-transfection, cells were harvested in the lysis buffer (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 5% glycerol, 0.5% NP-40, 5 mM NaF, 1 mM Na_3VO_4 and a protease inhibitor cocktail). The cell lysate was incubated with MBP or MBP-DUSP23 pre-bound to maltose-conjugated agarose matrix in the lysis buffer at 4°C for 4 h before washing three times with the lysis buffer. The proteins pulled down were analyzed by immunoblotting with FLAG mAb. Recombinant MBP fusion protein, MBP-DUSP23, was prepared using the pMAL-c2 expression system (NEB, Beverly, MA).

GCM1 stability analysis

To study the effect of DUSP23 on GCM1 stability, 293T cells were transfected with pGCM1-FLAG and increasing amounts of pDUSP23-Myc or pDUSP23DACS-Myc for 48 h. Cells were than harvested for immunoblotting using FLAG mAb. To study the effect of DUSP23 on GCM1 half-life, mock BeWo31 cells and BeWo31 cells expressing DUSP23-Myc- or DUSP23DACS-Myc were treated with cycloheximide for the indicated period of time and then harvested for immunoblotting with HA mAb. In a separate experiment, the three groups of BeWo31 cells were incubated under normoxic and hypoxic conditions for 24 h and then harvested for immunoblotting analysis of the HA-GCM1 protein level. Likewise, the three groups of BeWo31 cells incubated under normoxic and hypoxic conditions were subjected to immunoprecipitation with a phospho-specific antibody to Ser322 and immunoblotting with HA mAb to study the effect of DUSP23 on Ser322 dephosphorylation in GCM1. Preparation of the phospho-specific antibody to Ser322, p-Ser322-GCM1, has been previously described (15). Band intensities were analyzed using a Kodak DC290 zoom digital camera and 1D image analysis software.

In vitro GSK-3β phosphorylation and DUSP23 dephosphorylation of GCM1

Recombinant MBP fusion protein, MBP-GCM1 (167–349), which contained the amino acids 167–349 of GCM1, was prepared using the pMAL-c2 expression vector. As a control, the mutant MBP-GCM1 (167–349)AA, which harbored Ser322-to-Ala and Ser326-to-Ala mutations on the GSK-3β phosphorylation site in GCM1, was prepared. An amount of 1 μg of purified MBP, wild-type or mutant MBP-GCM1 (167–349) was incubated with 1 U recombinant GSK-3β (Sigma), 1 μCi γ-[^{32}P]-ATP and 0.2 mM ATP in the reaction buffer (25 mM MOPS, pH 7.2, 12.5 mM β-glycerophosphate, 25 mM $MgCl_2$, 5 mM EGTA, 2 mM EDTA, 25 mM DTT) at 30°C for 1 h. Recombinant DUSP23 and DUSP23DACS proteins with a C-terminal His tag, DUSP23-His and DUSP23DACS-His, were prepared using the pET21 expression vector. Similarly, recombinant DUSP23 with a C-terminal FLAG tag, DUSP23-FLAG, was prepared using the pET21 expression vector. The phosphatase activities of recombinant DUSP23 and DUSP23DACS proteins were confirmed by *in vitro* phosphatase assay using p-nitrophenyl phosphate (PNPP) as a substrate (data not shown). Dephosphorylation of GCM1 by DUSP23 was analyzed by incubation of the above-mentioned GSK-3β-treated wild-type or mutant MBP-GCM1 (167–349) with increasing amounts of recombinant DUSP23-His and DUSP23DACS-His, followed by SDS–PAGE electrophoresis and autoradiography.

Regulation of GCM1–DUSP23 interaction

To study regulation of GCM1–DUSP23 interaction by cAMP/PKA-signaling, BeWo cells were treated with or without 50 μM forskolin for 24 h and subjected to coimmunoprecipitation with GCM1 and DUSP23 antibodies. Phosphorylation of Ser269 and Ser275 in GCM1 by PKA was analyzed by immunoblotting with the p-Ser269275-GCM1 antibody, which was raised against a chemically synthesized phosphopeptide, YEKRKLS(PO_3)SSRTYS(PO_3)SGDL, in guinea pigs. For *in vitro* study of regulation of GCM1–DUSP23 interaction by PKA, 0.5 μg of MBP or MBP-GCM1(167–349) protein pre-bound to maltose-conjugated agarose matrix was incubated with 0.5 U PKA (Sigma) and 1 mM ATP in the reaction buffer (20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 12 mM $MgCl_2$) at 30°C for 30 min. After washing, the matrix was incubated with 0.1 μg of DUSP23-FLAG for pull-down assays.

Cell fusion analysis

Cell-cell fusion was analyzed based on a previously described co-culture assay system (6). Briefly, 293T cells were transfected with the red fluorescent protein plasmid, pDsRed1-N1 (Clontech, Mountain View, CA), for 24 h. To study the role of DUSP23 in placental cell fusion, the transfected 293T cells were trypsinized and co-cultured with BeWo cells expressing scramble or DUSP23 shRNA in six-well culture dishes, followed by treatment with or without 50 μM forskolin. After another 24 h at 37°C, cell fusions were examined under an Olympus microscope (Tokyo, Japan) equipped with a cooled charge-coupled device camera (DP50). Three microscopic fields per sample were randomly selected for examination in each of three independent experiments. Images were prepared for presentation using Adobe Photoshop® 6.0. Quantification of cell fusion was calculated as a fusion index of (N-S)/T, where N is the number of nuclei in the syncytia, S is the number of syncytia and T is the total number of nuclei counted.

Quantitative real-time PCR

RNA was isolated using RNeasy reagents (Qiagen, Hilden, Germany) and then transcribed into cDNA using SuperScript III reagents (Invitrogen) with an oligo-$(dT)_{20}$ primer. Quantification of the transcript levels of GCM1 target genes was preformed in the LightCycler system (Roche, Basel, Switzerland) using a commercial SYBR Green reaction reagent (Qiagen) and specific primer sets. The sequences of primer sets were 5′-TGGAACAACTTCAGCACAGA-3′ and 5′-GCCATTCA

AACAACGATAGG-3′ for syncytin-1, 5′-CGACTCAGT GTAAACAGCCA-3′ and 5′-CCACAGAAGCAAGACA AAGAAAAT-3′ for syncytin-2, 5′-GCAGAGGCCGGC ATTC-3′ and 5′-TCAGAGGTGGAAGTGGTACCCT-3′ for PGF, 5′-CTCCTGGCCATCATGCTCTC-3′ and 5′-G GCCACCAAGATGAGAAA-3′ for MFSD2, 5′-AACTC CATCATGAAGTGTGACG-3′ and 5′-GATCCACATC TGCTGGAAGG-3′ for β-actin and 5′-GCCATCAATG ACCCCTTCATT-3′ and 5′-TTGACGGTGCCATGGA ATTT-3′ for GAPDH.

RESULTS

Characterization of GCM1–DUSP23 interaction

To further understand the regulation of GCM1 activity, we identified DUSP23 as a GCM1-associated protein using a tandem affinity purification approach coupled with mass spectrometry (Supplementary Figure S1). We then investigated whether DUSP23 physically interacts with GCM1. To this end, 293T cells were transfected with pHA-GCM1 and pDUSP23-FLAG, followed by immunoprecipitation and immunoblotting with HA and FLAG monoclonal antibodies (mAbs). As shown in the left panel of Figure 1A, a specific interaction between HA-GCM1 and DUSP23-FLAG was detected. We further demonstrated that GCM1 interacts with DUSP23, but not DUSP22, which is a closely related DUSP (Supplementary Figure S2). The specific interaction between endogenous GCM1 and DUSP23 was confirmed in placental BeWo cells using DUSP23 and GCM1 antibodies for immunoprecipitation and immunoblotting, respectively (Figure 1A, middle). A direct interaction between GCM1 and DUSP23 was verified by the pull-down of recombinant DUSP23-FLAG proteins with MBP-GCM1, but not MBP (Figure 1A, right).

We next mapped the interaction domain of GCM1 for DUSP23. Recombinant MBP or MBP-DUSP23 proteins were incubated with cell lysates prepared from 293T cells transfected with pGal4-GCM1-FLAG expression plasmids encoding the Gal4 DNA-binding domain fused with either full-length or different truncated GCM1

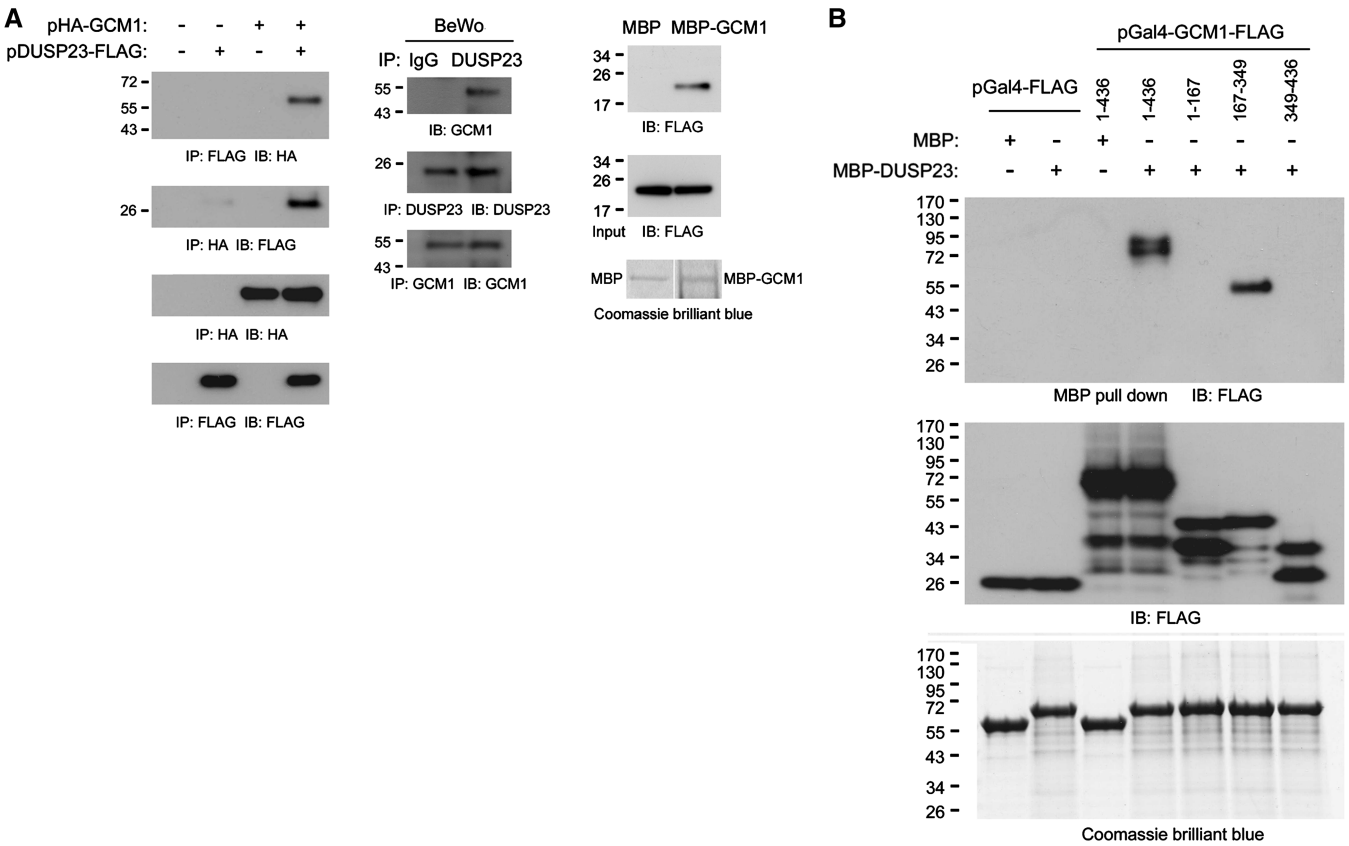

Figure 1. Characterization of the interaction between DUSP23 and GCM1. (**A**) Analysis of DUSP23-GCM1 interaction by coimmunoprecipitation. 293T cells were transfected with 1 μg of pHA-GCM1 and 1 μg of pDUSP23-FLAG for 48 h and then harvested for immunoprecipitation (IP) and immunoblotting (IB) with FLAG and HA mAbs. BeWo cells were subjected to immunoprecipitation with normal IgG or DUSP23 antibody, followed by immunoblotting with GCM1 antibody. Maltose-conjugated agarose matrix pre-loaded with 0.5 μg of recombinant MBP or MBP-GCM1 protein was incubated with 0.1 μg of recombinant DUSP23-FLAG for pull-down analysis and immunoblotting with FLAG mAb. (**B**) Identification of the DUSP23-interacting domain in GCM1. 293T cells were transfected with 1 μg of pGal4-FLAG, wild-type or deletion mutant pGal4-GCM1-FLAG expression plasmid. At 48 h post-transfection, cell extracts were prepared and incubated with maltose-conjugated agarose matrix pre-loaded with 2 μg of recombinant MBP or MBP-DUSP23 protein for pull-down analysis and immunoblotting with FLAG mAb. The middle panel is immunoblotting of Gal4-FLAG and Gal4-GCM1-FLAG proteins in the input cell extracts. The lower panel shows Coomassie brilliant blue staining of MBP and MBP fusion proteins in pull-down assays.

domains. As shown in Figure 1B, MBP-DUSP23, but not MBP, pulled down the full-length GCM1 and the GCM1 domain of amino acids 167–349. Of note, the immunoblotting analysis of input indicated that Gal4-GCM1-FLAG(1–436) proteins are prone to partial degradation. As a result, the intact and partially-degraded Gal4-GCM1-FLAG(1–436) proteins were coimmunoprecipitated with DUSP23. Overall, the DUSP23-interacting domain in GCM1 is localized to the region of amino acids 167–349, which also harbors the Ser322 phosphorylation site critical for GCM1 stability (15).

Stabilization of GCM1 by DUSP23

Because GCM1 stability can be regulated by phosphorylation, we tested the role of DUSP23 in regulation of GCM1 stability. To this end, 293T cells were cotransfected with pGCM1-FLAG, a GCM1 expression plasmid and pDUSP23-Myc or pDUSP23DACS-Myc, a wild-type or enzyme-dead DUSP23 expression plasmid. As shown in Figure 2A, the steady-state level of GCM1-FLAG increased in the presence of increasing amounts of wild-type DUSP23-Myc, but not the enzyme-dead DUSP23DACS-Myc. We also tested the effect of DUSP23 on GCM1-mediated transcriptional activation by transient expression experiments. The reporter plasmid, p(GBS)$_4$E1BLuc, which contains four copies of the GCM1-binding site, was cotransfected with pGCM1-FLAG and pDUSP23-Myc or pDUSP23DACS-Myc into 293T cells. GCM1-mediated transcriptional activation was significantly enhanced in the presence of the wild-type DUSP23-Myc in a dose-dependent manner (Figure 2B). Interestingly, the enzyme-dead DUSP23DACS-Myc was

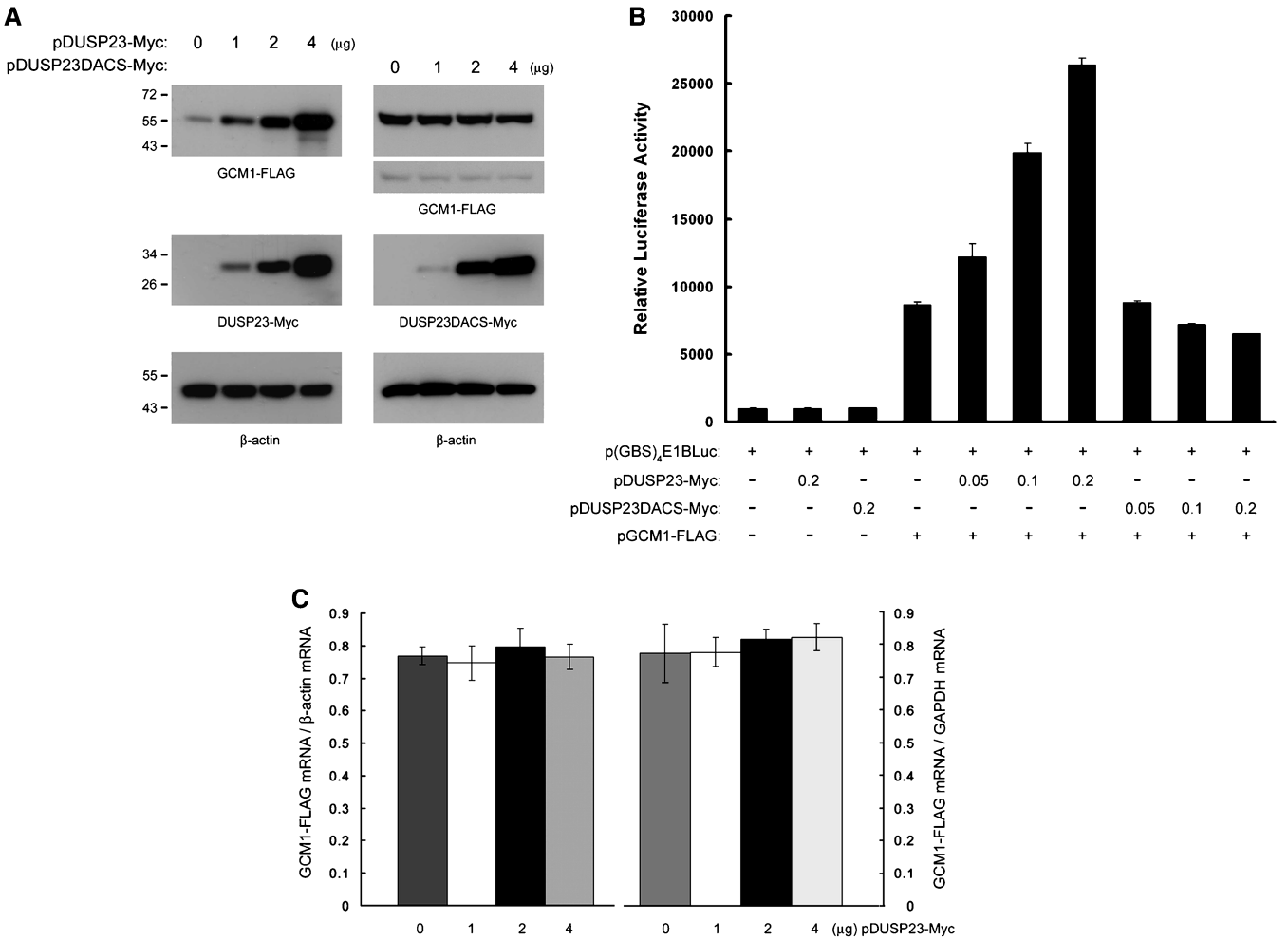

Figure 2. Regulation of GCM1 stability by DUSP23. (**A**) The steady state level of GCM1 increased in the presence of DUSP23. 293T cells were transfected with 1 μg of pGCM1-FLAG and the indicated amount of pDUSP23-Myc or pDUSP23DACS-Myc for 48 h. Cells were harvested for immunoblotted with FLAG, Myc or β-actin mAb. The result of a short exposure time (1 min) for the level of GCM1-FLAG in 293T coexpressing DUSP23-Myc is presented in the top of the left panel. Note that the result of a longer exposure time (5 min) for the level of GCM1-FLAG in 293T cell coexpressing DUSP23DACS-Myc is presented in the top of the right panel. For comparison, the result of a short exposure time (1 min) is provided underneath. (**B**) GCM1-mediated transcriptional activation is enhanced by DUSP23. 293T cells were transfected with 0.1 μg of p(GBS)$_4$E1BLuc and different combinations of 0.05 μg of pGCM1-FLAG and increasing amounts of pDUSP23-Myc or pDUSP23DACS-Myc for 48 h. Cells were then harvested for luciferase reporter assay. Mean values and the SD obtained from three independent experiments are presented. (**C**) The transcript levels of GCM1-FLAG in 293T cells coexpressing with different amounts of DUSP23-Myc were analyzed by quantitative real-time PCR. Mean values and the SD obtained from three independent experiments are presented.

able to counteract GCM1-mediated transcriptional activation (Figure 2B), suggesting that DUSP23DACS-Myc imposes a dominant-negative effect on GCM1 activity. To rule out the possibility that DUSP23 upregulates GCM1 transcription, we also measured the transcript level of GCM1-FLAG in transfected cells by quantitative real-time PCR. As shown in Figure 2C, the transcript level of GCM1-FLAG was normalized against β-actin or GAPDH and was comparable in cells expressing GCM1-FLAG with or without increasing amounts of DUSP23-Myc. These results suggested that the effect of DUSP23-Myc on elevation of GCM1-FLAG protein level is not due to differential expression of GCM1-FLAG transcript. Taken together, these results suggested that the phosphatase activity of DUSP23 is required to promote GCM1 stability in order to enhance the transcriptional activity of GCM1.

DUSP23 prevents GCM1 from ubiquitination and prolongs the half-life of GCM1

Because GCM1 is a labile protein subject to ubiquitination and proteasome-mediated degradation, we tested whether DUSP23 affects GCM1 ubiquitination. To this end, 293T cells were transfected with different combinations of pGCM1-FLAG, pHA-Ub, pDUSP23-Myc and pDUSP23DACS-Myc expression plasmids, followed by treatment with the proteasome inhibitor, MG132, to enrich the ubiquitinated GCM1-FLAG. As shown in Figure 3A, GCM1-FLAG was poly-ubiquitinated in the presence of HA-Ub. Interestingly, the level of poly-ubiquitinated GCM1-FLAG was significantly lower when DUSP23-Myc, but not DUSP23DACS-Myc, was coexpressed (Figure 3A). We further studied the effect of DUSP23 on the half-life of GCM1 in BeWo31 cells, which stably express HA-GCM1. BeWo31 cells were infected with lentivirus strains harboring empty, DUSP23-Myc, or DUSP23DACS-Myc expression cassettes. After antibiotic selection, stable clones were pooled and subjected to cycloheximide chase experiments. The half-life of HA-GCM1 in mock-infected BeWo31 cells was ~120 min, and was significantly prolonged in cells expressing DUSP23-Myc after chasing for 6 h (Figure 3B). Notably, the half-life of HA-GCM1 was shorter in BeWo31 cells expressing DUSP23DACS-Myc than in the mocked-infected BeWo cells (Figure 3B). These results suggest that DUSP23 increases GCM1 stability by preventing GCM1 ubiquitination.

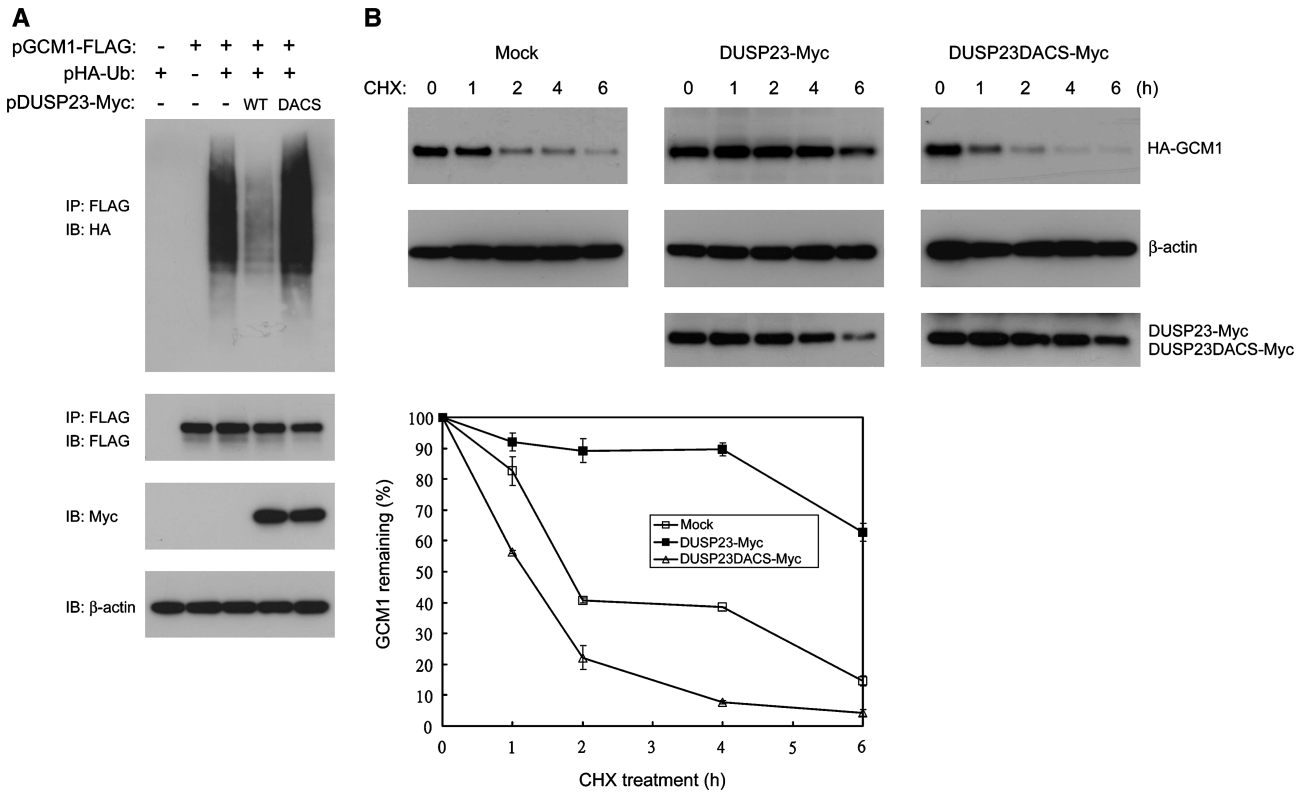

Figure 3. Regulation of GCM1 ubiquitination and half-life by DUSP23. (**A**) DUSP23 prevents GCM1 from ubiquitination. 293T cells were transfected with different combinations of 1 μg of pGCM1-FLAG, pHA-Ub, pDUSP23-Myc and pDUSP23DACS-Myc. At 24 h post-transfection, cells were treated with 40 μM MG132 for an additional 10 h, and then subjected to ubiquitination analysis by immunoprecipitation with FLAG mAb and immunoblotting with HA mAb. (**B**) DUSP23 prolongs the half-life of GCM1. Mock BeWo31 cells or BeWo31 cells stably expressing DUSP23-Myc or DUSP23DACS-Myc were treated with 75 μM cycloheximide (CHX) for the indicated period of time. The protein levels of HA-GCM1, DUSP23-Myc, DUSP23DACS-Myc and β-actin were then analyzed by immunoblotting with HA, Myc and β-actin mAbs. The band intensity of HA-GCM1 and β-actin was quantified by densitometric analysis. After normalization of HA-GCM1 with β-actin, the relative levels of HA-GCM1 proteins from two independent experiments were plotted against the time course of CHX treatment.

Dephosphorylation of Ser322 in GCM1 by DUSP23

Because Ser322 phosphorylation controls GCM1 ubiquitination and degradation, we tested whether DUSP23 stabilizes GCM1 via dephosphorylation of Ser322. Recombinant MBP-GCM1(167–349) was subjected to *in vitro* phosphorylation by GSK-3β and [^{32}P]-ATP. Specific phosphorylation of Ser322 in MBP-GCM1 (167–349) by GSK-3β was observed because the mutant MBP-GCM1(167–349)AA harboring a Ser-to-Ala mutation in Ser322 was not phosphorylated (Figure 4A). Labeled MBP-GCM1(167–349) was incubated with increasing amounts of recombinant DUSP23 or DUSP23DACS. As shown in Figure 4A, the level of Ser322-phosphorylated MBP-GCM1(167–349) was lower in the presence of DUSP23, but not DUSP23DACS. In addition, the effect of DUSP23 on Ser322-phosphorylated MBP-GCM1(167–349) was abolished by sodium orthovanadate (Na_3VO_4), a specific inhibitor of PTPs (Figure 4A). Therefore, DUSP23 was able to dephosphorylate Ser322 *in vitro*.

We next tested whether DUSP23 is involved in Ser322 dephosphorylation in placental BeWo31 cells stably expressing HA-GCM1. The mock, DUSP23-Myc-expressing and DUSP23DACS-Myc-expressing BeWo31 cells were subjected to normoxic or hypoxic conditions for 24 h. In accordance with our previous study that showed that hypoxia induces GCM1 degradation (15), the protein level of HA-GCM1 in mock-infected BeWo31 cells under hypoxia was decreased to 57.5% of that in cells under normoxia (Figure 4B, left). In contrast, HA-GCM1 was stabilized in cells expressing DUSP23-Myc under hypoxia (90.5% of normoxic cells, Figure 4B, middle). Interestingly, the protein level of HA-GCM1 was further decreased to 42.3% in cells expressing DUSP23DACS-Myc under hypoxia (Figure 4B, right). We then studied whether the observed stabilization effect of DUSP23 was correlated with dephosphorylation of Ser322 in GCM1. In a separate experiment, the BeWo31 cells were subjected to immunoprecipitation with a phospho-Ser322-specific antibody and then immunoblotting with HA mAb. As shown in the left panel of Figure 4C, consistent with our previous study (15), the level of Ser322-phosphorylated HA-GCM1 was much higher in mock-infected BeWo31 cells under hypoxia (438% relative to normoxia). Interestingly, Ser322-phosphorylated HA-GCM1 in the DUSP23-Myc-expressing BeWo31 cells under hypoxia could barely be detected and was similar to that in cells under normoxia (Figure 4C, middle). In contrast, the level of Ser322-phosphorylated HA-GCM1 was dramatically increased in the DUSP23DACS-Myc-expressing

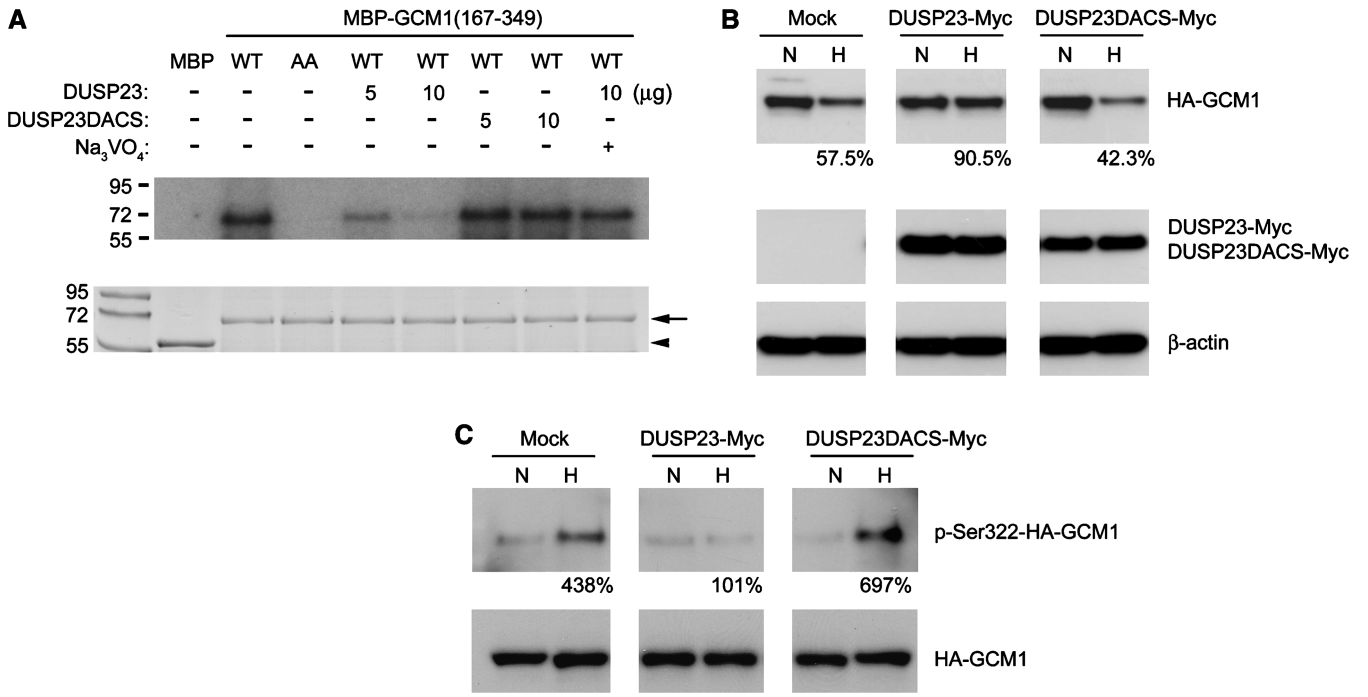

Figure 4. DUSP23 mediates Ser322 dephosphorylation in GCM1 *in vitro* and *in vivo*. (**A**) *In vitro* dephosphorylation of Ser322 by DUSP23. Recombinant MBP (arrowhead), MBP-GCM1(167–349) and MBP-GCM1(167–349)AA (arrow), bound on maltose matrix were incubated with recombinant GSK-3β proteins and [^{32}P]-ATP for *in vitro* kinase reactions at 30°C for 1 h. After washing, the reaction mixtures were incubated with increasing amounts of recombinant DUSP23-His or DUSP23DACS-His proteins in the presence or absence of 2 mM sodium orthovanadate (Na_3VO_4) at 37°C for 1 h before SDS–PAGE and autoradiography. (**B**) DUSP23 counteracts hypoxia-induced GCM1 degradation. Mock BeWo31 cells or BeWo31 cells stably expressing DUSP23-Myc or DUSP23DACS-Myc were incubated under normoxic and hypoxic conditions for 24 h and then subjected to immunoblotting with HA, Myc and β-actin mAbs. The numbers underneath indicate the ratios of the HA-GCM1 band intensity (normalized against β-actin) under hypoxia to that under normoxia. (**C**) DUSP23 suppresses hypoxia-induced Ser322 phosphorylation in placental cells. BeWo31 cells as described in (**B**) were cultured under normoxia or hypoxia in the presence of MG132 for 24 h, followed by immunoprecipitation with p-Ser322-GCM1 antibody and immunoblotting with HA mAb. The numbers underneath indicate the ratios of the Ser322-phosphorylated HA-GCM1 band intensity (normalized against HA-GCM1) under hypoxia to that under normoxia.

BeWo31 cells under hypoxia (697% relative to normoxia, Figure 4C, right). Therefore, the enzyme-dead DUSP23DACS is able to block DUSP23-mediated Ser322 dephosphorylation, which may underscore the observed shortening of GCM1 half-life by DUSP23DACS (Figure 3B). Taken together, these results suggested that DUSP23 mediates Ser322 dephosphorylation in GCM1 in placental cells.

Regulation of GCM1–DUSP23 interaction by cAMP/PKA signaling

Placental cell fusion is stimulated by activation of the cAMP/PKA-signaling pathway, which involves stabilization of GCM1 and activation of syncytin-1 gene expression (6,17,21,22). Together with the finding that GCM1 is stabilized by DUSP23, we next tested the effect of cAMP/PKA on the interaction between GCM1 and DUSP23. As shown in Figure 5A, the interaction between GCM1 and DUSP23 in BeWo cells was significantly enhanced by forskolin, which is a cAMP stimulant. Of note, the transcript and protein levels of DUSP23 in BeWo cells were not affected by forskolin (Figure 5A).

Our previous study indicated that Ser269 and Ser275 are both PKA phosphorylation sites (17). Correspondingly, phosphorylation of both sites was detected here after forskolin treatment using an antibody against the phosphorylated Ser269 and Ser275 in GCM1 (Figure 5A). Since both sites are in the DUSP23-interacting domain of GCM1, we tested whether phosphorylation of Ser269 and Ser275 by PKA enhances the interaction between GCM1 and DUSP23. To this end, recombinant MBP-GCM1(167–349) was subjected to *in vitro* PKA phosphorylation for phosphorylation of Ser269 and Ser275 (Figure 5B, middle). When non-phosphorylated and phosphorylated MBP-GCM1(167–349) were incubated with recombinant DUSP23-FLAG in pull-down assays, a significant increase of interaction between phosphorylated MBP-GCM1(167–349) and DUSP23-FLAG was detected (Figure 5B). Subsequently, we tested whether Ser269 and Ser275 phosphorylation correlates with increased interaction between GCM1 and DUSP23 in placental cells. BeWo and primary cytotrophoblast (CTB) cells were treated with or without forskolin, followed by immunoprecipitation and immunoblotting analysis. As shown in Figure 5C, forskolin stimulated Ser269 and Ser275 phosphorylation in both cell types. In addition, the PKA inhibitor H89 abolished the forskolin-induced GCM1 phosphorylation, supporting the position that PKA mediates Ser269 and Ser275 phosphorylation in placental cells. Correspondingly, the interaction between GCM1 and DUSP23 was enhanced in the presence of forskolin in both cell types, and the enhancement effect of forskolin was impaired by H89 (Figure 5C).

We further confirmed that Ser269 and Ser275 are critical for PKA in promoting GCM1–DUSP23 interaction by site-directed mutagenesis. 293T cells were transfected with different combinations of pDUSP23-FLAG, pHA-GCM1, pHA-GCM1SSAA (which encodes HA-GCM1 with Ser-to-Ala mutations in Ser269 and Ser275) and pPKAcata (which encodes the catalytic subunit of PKA), followed by treatment with or without forskolin. After coimmunoprecipitation analysis, the interaction between DUSP23-FLAG and HA-GCM1, but not HA-GCM1SSAA, was enhanced when the PKA catalytic subunit was coexpressed or in the presence of forskolin (Figure 5D). Furthermore, when both Ser269 and Ser275 were changed into glutamic acid, the resultant HA-GCM1SSEE exhibited higher binding activity with DUSP23 than the wild-type HA-GCM1 (Figure 5E). Taken together, these results suggest that activation of cAMP/PKA-signaling leads to Ser269 and Ser275 phosphorylation and enhanced interaction between GCM1 and DUSP23.

Regulation of trophoblastic fusion by DUSP23

We next tested whether DUSP23 is involved in the regulation of trophoblastic fusion. To this end, BeWo cells expressing scramble or DUSP23 shRNA were co-cultured with 293T cells expressing DsRed protein, followed by treatment with or without forskolin. As shown in Figure 6A, forskolin stimulated fusion between 293T cells expressing DsRed and BeWo cells expressing scramble shRNA (compare panels a and b, 0.26% versus 5.97% in terms of fusion index). Interestingly, the fusion activity of BeWo cells induced by forskolin was barely detected when DUSP23 was knocked down (compare panels b and d, 5.97% versus 0.49% in terms of fusion index). These results suggest that DUSP23 is required for the regulation of trophoblastic fusion by cAMP/PKA signaling.

We further studied the role of DUSP23 in regulation of GCM1 target gene expression using BeWo cells expressing scramble or DUSP23 shRNA, treated with or without forskolin, followed by quantitative real-time PCR analysis of the transcripts of GCM1 target genes including syncytin-1, syncytin-2, PGF and MFSD2 (syncytin-2 receptor). As shown in the left panel of Figure 6B, DUSP23 knockdown exerted a modest suppressive effect on the expression of syncytin-1 and syncytin-2 transcripts, but a stronger suppressive effect on the expression of PGF and MFSD2 transcripts. As expected, forskolin stimulated the expression of all four GCM1 target genes, which was correlated with an increase of GCM1 protein level (154% relative to mock scramble shRNA-expressing cells, compare lanes 1 and 3 in the right panel of Figure 6B). However, the stimulatory effect of forskolin on GCM1 target gene expression was suppressed when DUSP23 was knocked down, which was also correlated with a decrease in GCM1 protein level (154% versus 66%, compare lanes 3 and 4 in the right panel of Figure 6B). Taken together, these results suggest that DUSP23 is required for activation of the GCM1 transcriptional network by the cAMP/PKA-signaling pathway that leads to trophoblastic fusion.

Coordination of GCM1 dephosphorylation and acetylation in regulation of GCM1 stability

The observation that DUSP23 knockdown suppresses the positive effect of forskolin on GCM1 stability suggested that dephosphorylation of Ser322 may be a prerequisite

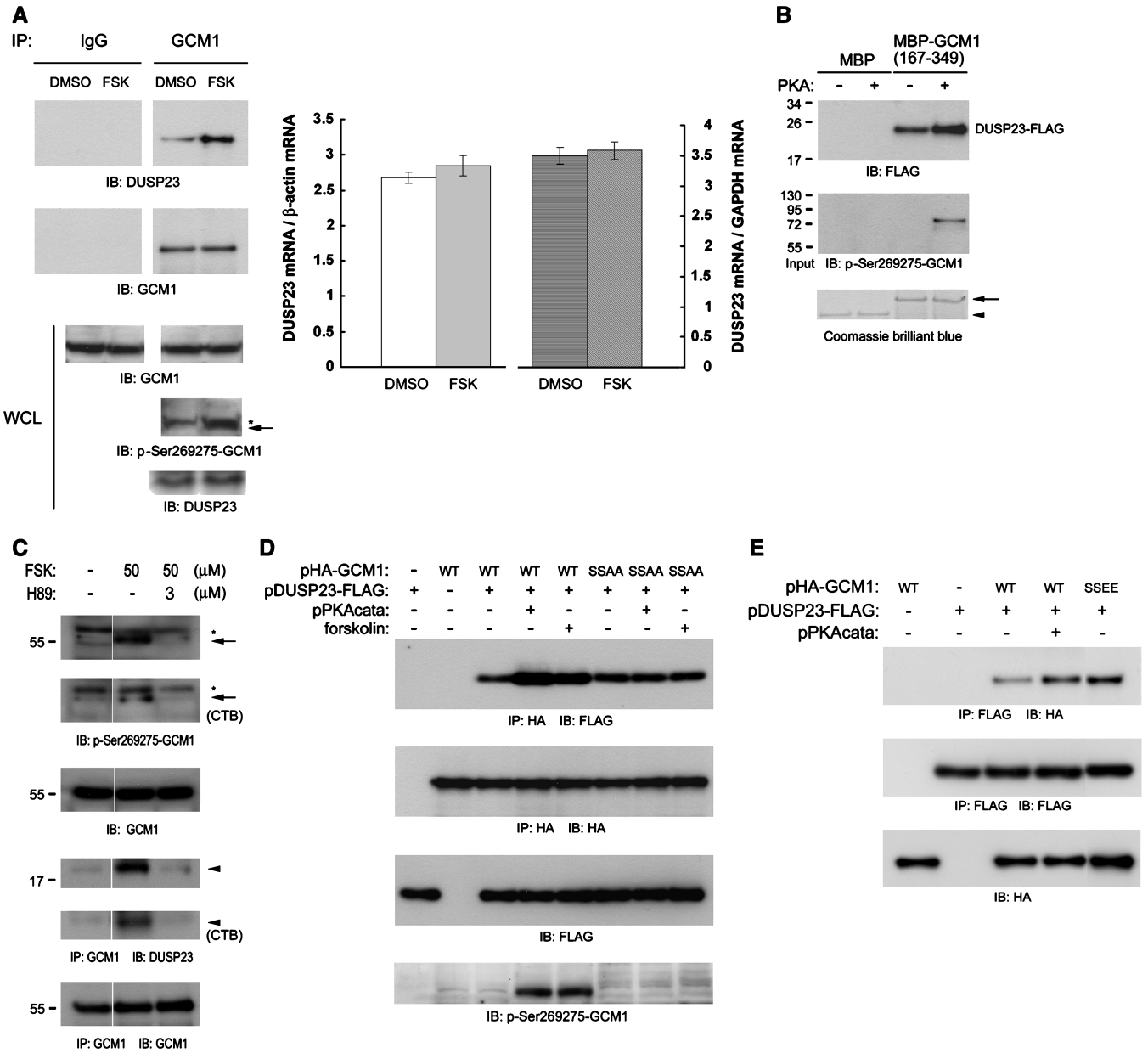

Figure 5. Regulation of GCM1 phosphorylation and GCM1–DUSP23 interaction. (**A**) Forskolin (FSK) stimulates the interaction between DUSP23 and GCM1. BeWo cells were treated with or without 50 μM forskolin for 24 h, followed by immunoprecipitation with normal IgG or GCM1 antibody and immunoblotting with DUSP23 and GCM1 antibodies, respectively. Note that specific interaction between GCM1 and DUSP23 was detected in immunoprecipitation with GCM1 antibody, but not normal IgG. The levels of Ser269- and Ser275-phosphorylated GCM1 and DUSP23 proteins in mock- or forskolin-treated BeWo cells were analyzed by immunoblotting with p-Ser269275-GCM1 and DUSP23 antibodies, respectively. Arrow and asterisk indicate the positions of the Ser269- and Ser275-phosphorylated GCM1 and a non-specific protein, respectively. In a separate experiment, cells were harvested for quantitative real-time PCR analysis of DUSP23 transcript. Mean values and the SD obtained from three independent experiments are presented. (**B**) Phosphorylation of Ser269 and Ser275 by PKA enhances DUSP23-GCM1 interaction. 0.5 μg of matrix-bound MBP or MBP-GCM1(167–349) was incubated with PKA for *in vitro* kinase reaction and then with 0.1 μg of recombinant DUSP23-FLAG in pull-down experiments. Phosphorylation of Ser269 and Ser275 in MBP-GCM1(167–349) was analyzed by immunoblotting with p-Ser269275-GCM1 antibody (middle panel). The lower panel is Coomassie brilliant blue staining of MBP (arrowhead) and MBP-GCM1(167–349) (arrow) in pull-down assays. (**C**) Activation of cAMP/PKA signaling stimulates Ser269 and Ser275 phosphorylation and enhances the interaction between GCM1 and DUSP23 in BeWo and primary cytotrophoblast cells. BeWo or CTB cells were treated with the indicated combinations of forskolin and H89. After 24 h incubation, cells were harvested for immunoblotting with p-Ser269275-GCM1 antibody. Arrow and asterisk indicate the positions of the Ser269- and Ser275-phosphorylated GCM1 and a non-specific protein, respectively. In a separate experiment, the cell lysates were subjected to immunoprecipitation with GCM1 antibody and immunoblotting with DUSP23 antibody. Arrowhead indicates the position of DUSP23. (**D**) Mutagenesis of Ser269 and Ser275 abolishes the enhancement effect of PKA and forskolin on DUSP23-GCM1 interaction. 293T cells were transfected with the indicated expression plasmids for 24 h, followed by treatment with or without 50 μM forskolin for additional 24 h. Cells were harvested for immunoprecipitation and immunoblotting with HA and FLAG mAbs. Note that coexpression of PKA or treatment with forskolin enhances the interaction between DUSP23 and the wild-type GCM1, but not the GCM1SSAA mutant. (**E**) Enhanced interaction between DUSP23 and phospho-mimetic GCM1 mutant, GCM1SSEE. 293T cells were transfected with the indicated expression plasmids for 24 h. Cells were harvested for immunoprecipitation and immunoblotting with HA and FLAG mAbs.

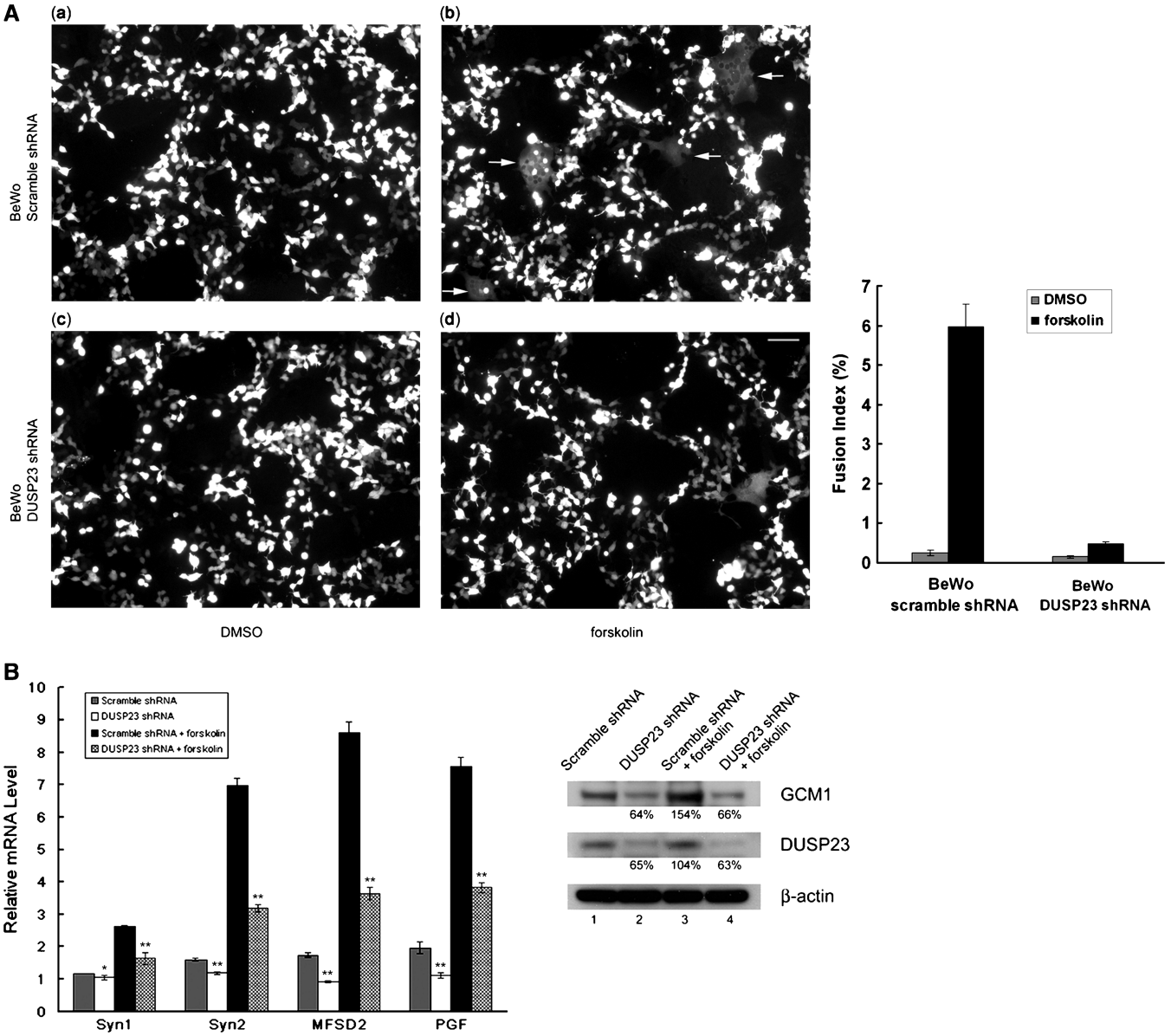

Figure 6. DUSP23 is required for cAMP/PKA-induced placental cell fusion. (**A**) DUSP23 knockdown suppresses placental cell fusion regulated by forskolin. 293T cells expressing the DsRed protein were co-cultured with BeWo cells expressing scramble or DUSP23-specific shRNA and treated with or without 50 μM forskolin for 24 h. Cell fusions were examined under a fluorescence microscope and analyzed by fusion index analysis. Arrows indicate syncytia in the forskolin-treated cells. Bar, 100 μm. Mean values and the SD obtained from three independent experiments are presented. (**B**) DUSP23 knockdown suppresses GCM1 target gene expression. BeWo cells expressing scramble or DUSP23 shRNA were treated with or without 50 μM forskolin for 24 h. Cells were then harvested for RNA purification and quantitative real-time PCR analysis with primer sets specific for the indicated GCM1 target genes (left). Syn1, syncytin-1; Syn2, syncytin-2. Mean values and the SD obtained from three independent experiments are presented. The Student's *t*-test was used to determine statistical significance for differences between means for scramble and DUSP23 shRNA-expressing cells. A *P*-value of <0.05 was considered significant (** = $P < 0.01$, * = $P < 0.05$). In a separate experiment, cells were harvested for immunoblotting analysis of GCM1, DUSP23 and β-actin with the indicated antibodies (right). The numbers underneath indicate the ratios of the GCM1 or DUSP23 band intensity (normalized against β-actin) relative to that in mock scramble shRNA-expressing cells.

for GCM1 acetylation in the cAMP/PKA-signaling pathway. We next investigated the potential interplay between GCM1 dephosphorylation and acetylation. We hypothesized that GCM1 dephosphorylation may facilitate subsequent CBP-mediated GCM1 acetylation to increase GCM1 stability and activity. Because acetylation of GCM1 by CBP protects GCM1 from ubiquitination, we first studied the interplay between DUSP23 and CBP in regulation of GCM1 ubiquitination in 293T cells transfected with different combinations of pGCM1-Myc, pGCM1K3R-Myc, pHA-Ub, pCBP-FLAG, pDUSP23-Myc and pDUSP23DACS-Myc expression plasmids. As shown in Figure 7A, CBP protected GCM1 from ubiquitination, which could be further enhanced by DUSP23 (lanes 4 and 5). However, this protection effect was counteracted by DUSP23DACS (compare lanes 4 and

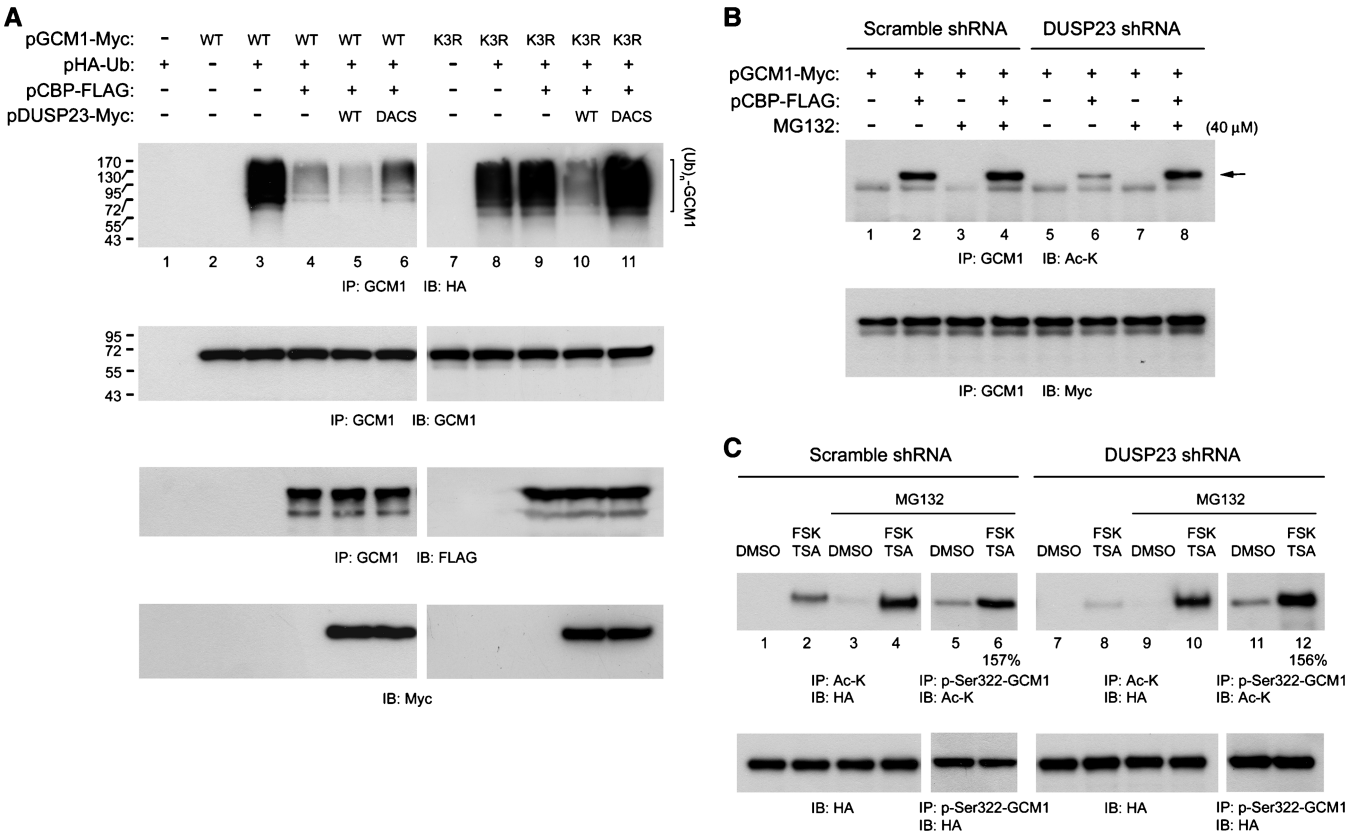

Figure 7. Coordination of GCM1 dephosphorylation and acetylation. (**A**) Interplay between DUSP23 and CBP in regulation of GCM1 ubiquitination. 293T cells were transfected with different combinations of 2 μg of pGCM1-Myc, 0.5 μg of pHA-Ub, 1 μg of pCBP-FLAG, 2 μg of pDUSP23-Myc and 2 μg of pDUSP23DACS-Myc. At 48 h post-transfection, cells were treated with 40 μM MG132 for an additional 6 h and then subjected to immunoprecipitation and immunoblotting with GCM1 antibody, and HA, FLAG and Myc mAbs. (**B**) DUSP23-mediated GCM1 dephosphorylation facilitates CBP-mediated GCM1 acetylation. 293T cells expressing scramble or DUSP23 shRNA were transfected with 2 μg of pGCM1-Myc and 1 μg of pCBP-FLAG. At 48 h post-transfection, cells were treated with 40 μM MG132 for an additional 10 h and then subjected to immunoprecipitation and immunoblotting with GCM1 antibody, and Ac-K and Myc mAbs. Arrow indicates the position of acetylated GCM1. (**C**) Dephosphorylation of GCM1 by DUSP23 facilitates GCM1 acetylation in placental cells. BeWo31 cells expressing scramble or DUSP23 shRNA were mock-treated or treated with 10 μM forskolin and 50 ng/ml TSA for 24 h for immunoprecipitation and immunoblotting with Ac-K and HA mAbs. In a separate experiment, cells were further treated with 20 μM MG132 for additional 10 h before harvesting for the above-described analysis. The numbers underneath lanes 6 and 12 indicate the ratios of the acetylated and Ser322-phosphorylated HA-GCM1 band intensity (normalized against Ser322-phosphorylated HA-GCM1) in FSK- and TSA-treated cells to that in mock-treated cells.

6 in Figure 7A). In agreement with our previous study (17), GCM1K3R, which harbors Lys-to-Arg mutations in the three major acetylation sites in GCM1, Lys367, Lys406 and Lys409, was ubiquitinated even in the presence of CBP. Although CBP failed to protect GCM1K3R from ubiquitination (lane 9, Figure 7A), GCM1K3R ubiquitination was dramatically decreased in the presence of wild-type DUSP23, but not DUSP23DACS (compare lanes 10 and 11 in Figure 7A). These results suggest that dephosphorylation of GCM1 by DUSP23 may enhance GCM1 acetylation by CBP and thereby protect GCM1 from ubiquitination.

We further studied whether GCM1 dephosphorylation by DUSP23 is a prerequisite step to CBP-mediated GCM1 acetylation. To this end, 293T cells expressing scramble or DUSP23 shRNA were transfected with pGCM1-Myc and pCBP-FLAG, followed by treatment with or without the proteasome inhibitor, MG132. As shown in Figure 7B, acetylation of GCM1 by CBP was significantly decreased in 293T cells expressing DUSP23 shRNA compared with that in 293T cells expressing scramble shRNA (compare lanes 2 and 6). Nevertheless, the negative effect of DUSP23 knockdown on CBP-mediated GCM1 acetylation was neutralized in the presence of MG132 as a similar level of acetylated GCM1 was detected in both cell types (compare lanes 4 and 8 in Figure 7B). We also tested the effect of DUSP23 on GCM1 acetylation in BeWo31 cells expressing scramble or DUSP23 shRNA after treatment with or without forskolin and the HDAC inhibitor, TSA. As shown in Figure 7C, GCM1 acetylation was evident in the presence of forskolin and TSA in BeWo31 cells expressing scramble shRNA, but barely detected in BeWo31 cells expressing DUSP23 shRNA (compare lanes 2 and 8). Again, the levels of acetylated GCM1 were comparable in both cell types when the cells were treated with MG132 (compare lanes 4 and 10 in Figure 7C). This was most likely due to MG132 inhibition of degradation of Ser322-phosphorylated GCM1 proteins and subsequent acetylation of the proteins by CBP. Indeed, this speculation was supported

by the observation that the increase of acetylated and Ser322-phosphorylated GCM1 protein level was comparable in both control and DUSP23-knockdown BeWo31 cells in the presence of MG132 (157% versus 156%, compare lanes 6 and 12 in Figure 7C). Taken together, these results suggest that GCM1 dephosphorylation by DUSP23 facilitates subsequent GCM1 acetylation by CBP.

DISCUSSION

Here we confirmed that DUSP23 is a functional phosphatase involved in GCM1 dephosphorylation and regulation of GCM1 activity in placenta. Several lines of evidence support this conclusion. First, GCM1 physically interacts with DUSP23. Domain mapping analysis further identified that the region of amino acids 167–349 in GCM1 is required for interaction with DUSP23. Second, DUSP23 is able to dephosphorylate Ser322 in GCM1 *in vitro* and in a stable cell line expressing HA-GCM1. Third, DUSP23 prevents GCM1 from ubiquitination and prolongs its half-life. As Ser322 phosphorylation is required for GCM1 ubiquitination and degradation, dephosphorylation of Ser322 by DUSP23 protects GCM1 from ubiquitination. Fourth, DUSP23 knockdown not only reduces GCM1 protein level, but also suppresses GCM1 target gene expression in placental cells. Correspondingly, the fusion activity of placental BeWo cells stimulated by PKA is significantly suppressed when DUSP23 is knocked down.

Protein phosphorylation mediated by kinases modulates metabolism, signal transduction, protein–protein interaction and protein degradation. In order to maintain cellular homeostasis, phosphorylated proteins are subjected to dephosphorylation by phosphatases. DUSPs, a family member of the type-I cysteine-based PTP superfamily, feature the ability to dephosphorylate tyrosine, serine and threonine residues. This dephosphorylation ability depends on the signature motif, HCXXXXXR, in the catalytic domains of DUSPs (19). Mitogen-activated protein kinase phosphatases (MKPs) are DUSPs with substrate specificity preference for one or more of the MAPKs: ERK, JNK and p38. Therefore, MKPs are critical regulators of MAPK signaling when MAPKs are phosphorylated and activated in response to extracellular stimuli (23). DUSP23 is a low molecular weight DUSP without an extended N-terminal domain found in MKPs. DUSP23 and other low molecular weight DUSPs, DUSP3 and DUSP22, are similar to the vaccinia virus VH1 phosphatase in terms of domain structure (19,24,25). Interestingly, VH1, DUSP3 and DUSP22 are involved in regulation of interferon and interleukin-signaling pathways by binding and dephosphorylating STAT proteins (26–28). It is not known whether DUSP23 has similar effects on STAT proteins.

Previous studies have provided conflicting results indicating that DUSP23 may dephosphorylate ERK or activate JNK and p38 in different cell types (25,29). Nevertheless, in the present study we not only identified GCM1 as a *bona fide* biological substrate for DUSP23, but also revealed a novel biological function for DUSP23 in regulation of placental cell fusion by controlling GCM1 dephosphorylation and stability. We mapped the interaction domain of GCM1 for DUSP23 to the region of amino acids 167–349, which also harbors the Ser322 residue critical for GCM1 stability. However, the interaction between GCM1 and DUSP23 seems to be independent of Ser322 phosphorylation and the phosphatase activity of DUSP23 as both recombinant DUSP23 and DUSP23DACS bind efficiently to non-phosphorylated MBP-GCM1(167–349) (Supplementary Figure S3A). In addition, our *in vitro* studies indicated that DUSP23 dephosphorylates Ser322, but not Ser269 and Ser275 (Supplementary Figure S3B), suggesting that dephosphorylation of Ser269 and Ser275 is mediated by other phosphatase. As Ser269 and Ser275 are within the DUSP23-interacting domain in GCM1 (amino acids 167–349), it will be instructive to investigate how phosphorylation of Ser269 and Ser275 in GCM1 facilitates its interaction with DUSP23.

That DUSP23-mediated Ser322 dephosphorylation plays an important role in regulation of GCM1 stability is supported by the finding that DUSP23 and DUSP23DACS are able to decrease and increase GCM1 ubiquitination, respectively (Figure 3A). Accordingly, the half-life of GCM1 is increased and decreased by DUSP23 and DUSP23DACS, respectively (Figure 3B). In addition, our recent study indicates that the mutant GCM1S322A, which harbors a Ser322-to-Ala mutation, is resistant to ubiquitination and very stable (15). Because Ser322 phosphorylation is required for FBW2 recognition and Ser322 is no longer available in GCM1S322A, its stabilization is very likely due to prevention of FBW2-mediated ubiquitination, instead of Ser322 dephosphorylation by DUSP23. Collectively, these findings also support that the intracellular fate of Ser322-phosphorylated GCM1 is under control of FBW2 and DUSP23.

Recent studies have demonstrated that the interplay between different types of post-translational modification is important in the regulation of protein activity and signal transduction. A phosphorylation–acetylation switch is involved in regulation of STAT1 signaling, such that STAT1 acetylation by CBP facilitates its dephosphorylation by TCP45 phosphatase and thereby suppresses the STAT1 signaling (30). On the other hand, CDC6, which is a regulator of DNA replication, is acetylated by GCN5 and is then susceptible to phosphorylation by cyclin A-CDKs in early S phase. These sequential modifications facilitate translocation of CDC6 to the cytoplasm for proper S-phase progression (31). By analogy, an interplay between different types of post-translational modification of GCM1 may provide precise fine-tuning of GCM1 activity in regulation of placental development and function. Indeed, the present study has demonstrated that Ser322 dephosphorylation in GCM1 by DUSP23 facilitates GCM1 acetylation by CBP in the cAMP/PKA-signaling pathway.

The coordination of GCM1 dephosphorylation and acetylation is of physiological significance. Because syncytiotrophoblast layer undergoes apoptosis and sheds

into the circulation, replenishment of new syncytiotrophoblast layer is expected to be tightly controlled through regulation of GCM1 stability and activity. In this scenario, phosphorylation of Ser322 in GCM1 by GSK-3β and dephosphorylation of Ser322 by DUSP23 may respectively serve as a negative and a positive regulator maintaining proper GCM1 activity in placenta. When exposed to pathological hypoxia, over-activated GSK-3β promotes Ser322 phosphorylation; consequently, GCM1 is ubiquitinated and degraded leading to breakdown of the GCM1 transcriptional network in preeclampsia (15). Nevertheless, Ser322-phosphorylated GCM1 is rescued from degradation by DUSP23 in order to promote trophoblastic fusion when new syncytiotrophoblast layer is needed to meet the physiological demands. In this regard, activation of cAMP/PKA-signaling leads to phosphorylation of Ser269 and Ser275 in GCM1, which enhances the recruitment of DUSP23 to mediate Ser322 dephosphorylation and increase the protein level of GCM1. Finally, CBP mediates GCM1 acetylation to further stabilize GCM1 and the CBP-GCM1 complex synergistically transactivates syncytin and syncytin receptor expression to facilitate trophoblastic fusion. Collectively, our study revealed a novel physiological function for DUSP23 in control of trophoblastic fusion via GCM1 dephosphorylation. Our study also revealed that coordinated GCM1 phosphorylation, dephosphorylation and acetylation are involved in cAMP/PKA-induced GCM1 activation and trophoblastic fusion.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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